Sall genes regulate region-specific morphogenesis in the mouse limb by modulating Hox activities

Yasuhiko Kawakami[1,2,3,4], Yukako Uchiyama[5], Concepcion Rodriguez Esteban[1], Toshiaki Inenaga[5], Naoko Koyano-Nakagawa[2,4,6], Hiroko Kawakami[1,2,3], Merce Marti[7], Marie Kmita[8], Paula Monaghan-Nichols[9], Ryuichi Nishinakamura[5] and Juan Carlos Izpisua Belmonte[1,7,*]

The genetic mechanisms that regulate the complex morphogenesis of generating cartilage elements in correct positions with precise shapes during organogenesis, fundamental issues in developmental biology, are still not well understood. By focusing on the developing mouse limb, we confirm the importance of transcription factors encoded by the Sall gene family in proper limb morphogenesis, and further show that they have overlapping activities in regulating regional morphogenesis in the autopod. *Sall1/Sall3* double null mutants exhibit a loss of digit1 as well as a loss or fusion of digit2 and digit3, metacarpals and carpals in the autopod. We show that Sall activity affects different pathways, including the Shh signaling pathway, as well as the Hox network. Shh signaling in the mesenchyme is partially impaired in the *Sall* mutant limbs. Additionally, our data suggest an antagonism between Sall1-Sall3 and Hoxa13-Hoxd13. We demonstrate that expression of *Epha3* and *Epha4* is downregulated in the *Sall1/Sall3* double null mutants, and, conversely, is upregulated in *Hoxa13* and *Hoxd13* mutants. Moreover, the expression of *Sall1* and *Sall3* is upregulated in *Hoxa13* and *Hoxd13* mutants. Furthermore, by using DNA-binding assays, we show that Sall and Hox compete for a target sequence in the *Epha4* upstream region. In conjunction with the Shh pathway, the antagonistic interaction between *Hoxa13-Hoxd13* and *Sall1-Sall3* in the developing limb may contribute to the fine-tuning of local Hox activity that leads to proper morphogenesis of each cartilage element of the vertebrate autopod.

INTRODUCTION

The development of the vertebrate limb has long served as an experimental and conceptual model system with which to study a variety of biological processes (reviewed by Capdevila and Izpisua Belmonte, 2001; Niswander, 2003; Tabin and Wolpert, 2007). Numerous studies have identified several secreted cell-cell signaling molecules, such as members of the *Wnt*, fibroblast growth factor (*Fgf*) and hedgehog gene families, responsible for a variety of processes during limb development. For example, embryonic and genetic studies have demonstrated the role of the *Wnt* family in the initiation of limb development (Kawakami et al., 2001), the role of Fgf genes as an instructive factor for proximal-distal patterning (Mariani et al., 2008), and the role of sonic hedgehog (*Shh*) in anterior-posterior patterning (Riddle et al., 1993).

These signaling pathways cooperate with one another to sustain limb outgrowth. For instance, Shh, produced in the zone of the polarizing activity (ZPA), regulates gremlin 1 (*Grem1*) expression in the posterior mesenchyme (Capdevila et al., 1999; Panman et al., 2006). Grem1-mediated BMP antagonism is crucial for maintenance of the expression of Fgfs in the apical ectodermal ridge (AER) (Khokha et al., 2003; Michos et al., 2004). FGF proteins, secreted from the AER, act on underlying mesenchyme to promote cell survival (Dudley et al., 2002), and in the posterior mesenchyme to maintain *Shh* expression (Laufer et al., 1994). Such a feedback loop mediates distal outgrowth, and, thus, proper formation of the autopod (Scherz et al., 2004). Shh-mediated counteraction of the Gli3 repressor also regulates the anterior-posterior patterning of digits (Litingtung et al., 2002; te Welscher et al., 2002). Although numerous studies have focused on the role of such signaling pathways and their interactions, the specific mechanisms that regulate region-specific morphogenesis, which leads to a stereotyped morphology of each limb skeletal element, remain elusive.

Human syndromes provide an opportunity to identify novel genes involved in limb development and have indeed given us invaluable clues in understanding the molecular and genetic bases of limb development (Wilkie, 2003). One such gene identified from human diseases is the *SALL1* gene, one of the four SALL genes in humans and mice, which are related to the *Drosophila spalt* gene. *SALL1* encodes a multi-zinc finger domain transcription factor (Nishinakamura and Osafune, 2006; Sweetman and Munsterberg, 2006), and mutations in the *SALL1* gene cause Townes-Brocks syndrome (TBS) (Kohlhase et al., 1998). Individuals with TBS exhibit multiple defects, including limb alterations. The human TBS disorders are mainly due to a dominant-negative action of the truncated SALL1 protein, though milder phenotypes have been reported from haploinsufficiency of *SALL1* (Borozdin et al., 2006; Kohlhase, 2000). In mice, no limb defects are reported in *Sall1* knockout or heterozygous mice (Nishinakamura et al., 2001). Conversely, two mouse models producing truncated Sall1 protein have shown some TBS-like phenotypes in the limb (Kiefer et al., 2003; Kiefer et al., 2008). These reports, together with a biochemical analysis demonstrating

[1]Gene Expression Laboratory, The Salk Institute for Biological Studies, 10010 N. Torrey Pines Road, La Jolla, CA 92037, USA. [2]Stem Cell Institute, University of Minnesota, 2001 6th SE, Minneapolis, MN. 55455, USA. [3]Department of Genetics, Cell Biology and Development, 6-160 Jackson Hall, 321 Church St. SE, Minneapolis, MN 55455, USA. [4]Developmental Biology Center, University of Minnesota, 321 Church St. SE, Minneapolis, MN 55455, USA. [5]Division of Integrative Cell Biology, Institute of Molecular Embryology and Genetics, Global COE 'Cell Fate Regulation Research and Education Unit', Kumamoto University, Kumamoto, Japan 860-0811. [6]Department of Neuroscience, University of Minnesota, 321 Church St. SE, Minneapolis, MN 55455, USA. [7]Center of Regenerative Medicine in Barcelona, Doctor Aiguader, 88, 08003 Barcelona, Spain. [8]Laboratory of Genetics and Development, Institut de Recherches Cliniques de Montréal (IRCM), Université de Montréal, 110 avenue des Pins Ouest, H2W 1R7, Montréal, Quebec, Canada. [9]Department of Neurobiology, University of Pittsburgh, Pittsburgh, PA 15261, USA.

*Author for correspondence (e-mail: belmonte@salk.edu)

Accepted 8 December 2008

that truncated Sall1 protein can form complexes with all Sall proteins (Kiefer et al., 2003), suggest that the truncated Sall1 protein inhibits other Sall family proteins, leading to the TBS phenotypes. However, no limb phenotype has been reported to date in mice lacking other Sall genes by conventional knockout approaches. *Sall2* is dispensable for embryonic development (Sato et al., 2003). *Sall3*-null mice exhibit a cleft palate, but no limb defects are observed (Parrish et al., 2004). *Sall4* mutants die at the peri-implantation stage, making it difficult to evaluate its role in organogenesis (Elling et al., 2006; Sakaki-Yumoto et al., 2006; Warren et al., 2007). Interestingly, a genetic interaction between *Sall1* and *Sall4* is needed for the proper development of several organs (Sakaki-Yumoto et al., 2006), suggesting functional redundancy between Sall genes.

Recent reports suggest a functional interaction between Sall and Hox genes during development in invertebrates. For example, *spalt*, the invertebrate homolog of Sall, acts in combination with Hox genes in *Drosophila* embryos to specify segmental identities (Copf et al., 2006). During wing/haltere development, *spalt* is regulated by a Hox gene, *Ubx* (Galant et al., 2002). In *C. elegans*, a *spalt* homolog, *sem-4*, directly regulates the expression of Hox genes, *elg-5* and *lin-39*, in touch receptor specification and vulval development, respectively (Grant et al., 2000; Toker et al., 2003). In the crustacean *Artemia*, *spalt* represses a Hox gene during the morphogenesis of trunk segments (Copf et al., 2006). Therefore, functional interactions between *spalt* and Hox genes have important roles in many aspects of invertebrate development.

In vertebrates, Hox proteins are crucial for limb development (reviewed by Zakany and Duboule, 2007). Hox genes encode transcription factors and in the mammalian genome the 39 genes are organized as 13 paralogs into four clusters (*Hoxa*, *Hoxb*, *Hoxc* and *Hoxd*) (Pearson et al., 2005), of which *Hoxa* and *Hoxd* are crucial for proper limb development (Kmita et al., 2005). *Hoxa* and *Hoxd* genes, which are located at the 5′ extremity of their respective clusters (so called 5′ Hox genes) are necessary for proper development of digits (Zakany et al., 1997). Human mutations have also highlighted the importance of HOX genes in human limb development. Hand-foot-genital syndrome is caused by mutations in the *HOXA13* gene, and synpolydactyly type II is caused by mutations in the *HOXD13* gene (Goodman, 2002; Lappin et al., 2006). In gene targeting experiments, *Hoxa13* and *Hoxd13* mutant mice each exhibit distinct phenotypes affecting autopod development (Fromental-Ramain et al., 1996). Mice with compound mutations in the *Hoxa13* and *Hoxd13* genes exhibit complex and more severe phenotypes, suggesting distinct and redundant functions of these two crucial *Hox13* paralogous genes. Furthermore, misexpression experiments in chick and mouse embryos have demonstrated that *Hoxa13* and *Hoxd13* regulate region-specific morphogenesis of cartilage elements in the autopod (Goff and Tabin, 1997; Williams et al., 2006; Yokouchi et al., 1995). Despite all of these advances, our understanding of how Hox genes specifically control region-specific morphogenesis in the limb is still unclear.

Based on the human TBS limb phenotypes and on the lack of limb defects in mice with individual Sall knockouts, we speculated that during limb development, Sall genes might have redundant activities that can only be identified by the study of compound mutants. We have analyzed *Sall1;Sall3* allelic series, and demonstrate that *Sall1* and *Sall3* have partially redundant activity. Our analyses suggest that Sall genes are involved in the Shh signaling, as well as in Shh-independent processes. We further show evidence that Sall and Hox activities are mutually antagonistic in the autopod, and that this

antagonism may contribute to a fine-tuning of local Hox activity that leads to proper morphogenesis of each cartilage element of the vertebrate autopod.

MATERIALS AND METHODS
Mouse mutants
Sall1 and *Sall3* mutants have been described previously (Nishinakamura et al., 2001; Parrish et al., 2004). As *Sall1/Sall3* double heterozygous mice were almost infertile when assessed by natural mating; all of the progeny were obtained by in vitro fertilization, which lead to the efficient production of compound mutant embryos. *Hoxa13* and *Hoxd13* mutants have been described previously (Fromental-Ramain et al., 1996; Kmita et al., 2000). *Shh* mutants (Chiang et al., 1996), *Gli3* mutants (Buscher et al., 1998) and *Tbx5* mutants (Bruneau et al., 2001) have been previously described. Skeletal samples were examined as described (McLeod, 1980).

In situ hybridization
Whole-mount in situ hybridization was performed following a standard protocol (Wilkinson, 1993).

Electrophoretic mobility shift assay (EMSA)
HA-Sall1, *Flag-Hoxa13* and *Flag-Hoxd13* were cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) and translated in vitro by using the TNT T7 system (Promega, Madison, WI.) according the manufacturers' instruction. The double-strand probes corresponding to –2028 to –2001 (transcription starting site as 1 with the NM_007936 as cDNA sequence) of the mouse *Epha4* gene contains the following sequences: wt probe, CGCGGT-TATTTTTAATAATTTATGCACA; mutant 1, CGCGGTTATTTTTAAT-cATTgATGCACA; mutant 2, CGCGGggcgTTTTAATAATTTATGCA-CA; mutant 3, CGCGGggcgTTccgATcAcTgATGCACA. (Lower case letters indicate mutations.)

EMSA was performed following a standard protocol (Yoh and Privalsky, 2001). Anti-HA (Covance, 16B12, Emeryville, CA) and anti-Flag (Sigma, M2, St Louis, MO) antibodies were used.

Luciferase reporter assay
A mouse *Epha4* upstream region (–2110 to –1980) that contains the sequence analyzed in the EMSA assay was subcloned into pGL3 (Promega) with the thymidine kinase promoter (TK). pRL-TK (Promega) was used as an internal control. NIH3T3 cells were transfected with the Epha4-TK-Luciferase, pRL-TK and various combinations of expression plasmids carrying *Sall1*, *Hoxa13* or *Hoxd13* by using Fugene6 (Roche, Indianapolis, IN), according to the manufacturer's instructions. Forty-eight hours after transfection, cells were subjected to analysis using the Dual-Luciferase Reporter Assay System (Promega). Results were expressed as fold increase compared with samples with an empty vector. Experiments were performed in triplicate, and statistical significance is analyzed by ANOVA followed by Tukey's comparison.

RESULTS
Combined activity of *Sall1* and *Sall3* contributes to the development of the autopod
SALL1 mutations in humans cause TBS, which results in limb defects (Kohlhase et al., 1998). The fact that no limb defects are reported in mice lacking *Sall1*, *Sall2*, *Sall3* or *Sall4* suggested a functional redundancy between Sall genes (Nishinakamura et al., 2001; Parrish et al., 2004; Sato et al., 2003). Among those Sall genes expressed in limb buds (Buck et al., 2001; Kohlhase et al., 2002; Ott et al., 2001), we focused on *Sall1* and *Sall3* double mutants, as early lethality of *Sall4*$^{-/-}$ embryos prevented the analysis of limb development in absence of *Sall4* function.

To gain insights into the respective contribution of *Sall1* and *Sall3* genes, we generated *Sall1/Sall3* allelic series and analyzed skeletons at E15.5. We did not obtain *Sall1*$^{-/-}$;*Sall3*$^{-/-}$ embryos at older stages, probably because loss of both *Sall1* and *Sall3* leads to lethality. The reason for the lethality is unknown at this point; however, E15.5

skeletons provided us with information to investigate the requirement of *Sall1* and *Sall3* during mouse limb development. Although the stylopod and zeugopod of all *Sall1;Sall3* mutants appear normal, we observed defects in the autopod both in the forelimb and hindlimb at E15.5 (Fig. 1; data not shown). We observed a fusion or lack of carpal elements, as well as fusion of metacarpal elements. $Sall1^{-/-};Sall3^{+/+}$ mutants show a mild fusion phenotype between metacarpal elements for digit4 and digit5 at a very proximal region (Fig. 1E). $Sall1^{-/-};Sall3^{+/-}$ mutants exhibit a more severe phenotype, as shown by the gross fusion in the metacarpal elements for digit2 and digit3, and those for digit4 and digit5, in addition to a loss of digit1 (Fig. 1F). Finally, $Sall1^{-/-};Sall3^{-/-}$ mutants exhibit further small carpal elements, more severe metacarpal fusion and a loss of digit1, and loss or fusion of digit2 and digit3 (Fig. 1G). Conversely, in the $Sall1^{+/-};Sall3^{+/-}$ and $Sall1^{+/-};Sall3^{-/-}$ mutants, we did not observe these defects (Fig. 1B,D), indicating that a single allele of the *Sall1* gene is sufficient for proper limb development.

These results indicate that *Sall1* and *Sall3* are partially redundant, but not equivalent. *Sall1* can compensate for the loss of *Sall3*, whereas *Sall3* can only partially compensate for the loss of *Sall1* based on minor defects in the carpal elements observed in $Sall1^{-/-}$ autopods. Together, our data indicate that a combined activity of *Sall1* and *Sall3* contributes to the proper formation of the autopod.

Expression of *Sall1* and *Sall3* is regulated by the Shh-Gli3 pathway in the developing limb

The $Sall1^{-/-};Sall3^{-/-}$ mutant limb exhibited defects in the autopod. Progression of limb development and formation of the autopod requires Shh-mediated counteraction of Gli3 (Litingtung et al., 2002; te Welscher et al., 2002). Previous experiments in chicks suggested that *Sall1* might be involved in distal limb patterning and that this putative function involves Shh signaling (Capdevila et al., 1999; Farrell and Munsterberg, 2000). Furthermore, it has been recently shown that reduced *Shh* signaling preferentially affects the formation of digit3 (Scherz et al., 2007; Zhu et al., 2008). In our study, we also observed that *Sall1;Sall3* inactivation predominantly disrupted the formation of digit3 (Fig. 1), consistent with the

possibility that the function of *Sall1* and *Sall3* is linked to Shh signaling. As several factors involved in the Shh pathway are regulated by Shh signaling itself (Zuniga et al., 1999), we first analyzed whether *Sall1* and *Sall3* expression is regulated by Shh and Gli3. The endogenous expression of *Sall1* and *Sall3* at E10.5 is restricted to the distal mesenchyme and is posteriorly biased (Fig. 2A,D). In the $Shh^{-/-}$ limb, both genes are severely downregulated (Fig. 2B,E), indicating that Shh signaling is required for expression of *Sall1* and *Sall3*. By contrast, expression of both genes is expanded towards the anterior in the $Gli3^{-/-}$ limb (Fig. 2C,F), indicating that Gli3 signaling negatively regulates expression of *Sall1* and *Sall3*. These results suggest that the Shh-Gli3 pathway impacts upon *Sall1* and *Sall3* expression at early stages of limb development.

Reduced Shh signaling in the *Sall1;Sall3* mutant limb

To further examine whether Sall activity is involved in Shh signaling, we monitored the expression of several key genes downstream of *Shh* that are required for normal limb outgrowth. Although *Shh* expression in the ZPA and *Fgf8* expression in the AER appear normal at E10.5 (see Fig. S1 in the supplementary material), we observed an alteration in *Grem1* expression (Fig. 3A,B), which is known to be regulated by the Shh-BMP pathway in the posterior mesenchyme (Capdevila et al., 1999; Merino et al., 1999; Nissim et al., 2006; Panman et al., 2006). We detected wild-type *Grem1* expression in a wide region of the posterior mesenchyme. By contrast, *Grem1* expression was weaker and restricted to a smaller region in the $Sall1^{-/-};Sall3^{-/-}$ limb (Fig. 3A,B). This was also evident in the E11.5 mutant limb (Fig. 3C,D). These results suggest reduced Shh signaling in the absence of *Sall1* and *Sall3*. Despite these changes, it seems that Sall activity is not required in the entire limb mesenchyme, as the skeletal phenotype is restricted to the autopod (Fig. 1), consistent with *Sox9* expression at E11.5 (Fig. 3E,F). Reflecting the defects at E15.5, we observed loss of digit1 and fusion of digit2 and digit3 primordia at E11.5 (Fig. 3E,F). Segregation of digit4 and digit5 primordia was also delayed. Correlating with the defects, the anterior and posterior margin of the *Fgf8* expression domain in the AER is shorter in the mutant than in
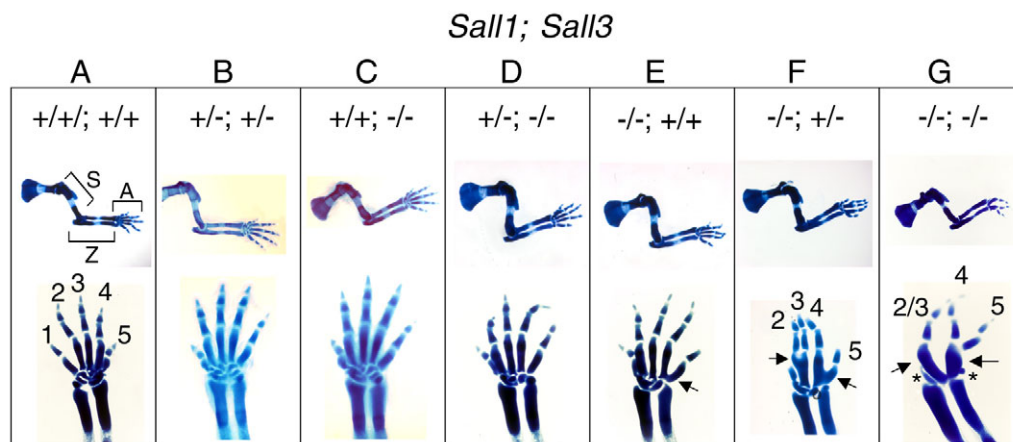
Fig. 1. Combined activity of *Sall1* and *Sall3* contributes to the development of the autopod. Alcian Blue-stained E15.5 forelimbs of *Sall1;Sall3* mutants are shown. Genotypes of *Sall1;Sall3* are indicated on the top: (**A**) +/+;+/+, (**B**) +/−;+/−, (**C**) +/+;−/−, (**D**) +/−;−/−, (**E**) −/−;+/+, (**F**) −/−;+/− and (**G**) −/−;−/−. Middle panels show lateral views of entire forelimb skeletons, and the bottom panels show dorsal views of the autopod. In A, the stylopod, zeugopod and autopod are indicated as S, Z and A, and digits are indicated with 1-5. Metacarpal fusions in the $Sall1^{-/-};Sall3^{+/+}$ (E), $Sall1^{-/-};Sall3^{+/-}$ (F) and $Sall1^{-/-};Sall3^{-/-}$ (G) mutants are indicated by arrows. Two small carpal elements left in the $Sall1^{-/-};Sall3^{-/-}$ (G) mutant are indicated by asterisks. Skeletal phenotypes become more severe from the left ($Sall1^{+/+};Sall3^{+/+}$; A) to the right ($Sall1^{-/-};Sall3^{-/-}$; G).
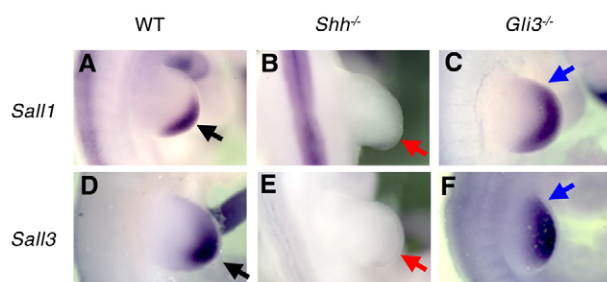
Fig. 2. Expression of *Sall1* and *Sall3* is regulated by Shh-Gli3. Dorsal views of E10.5 forelimbs stained with *Sall1* (**A-C**) and *Sall3* (**D-F**) with the anterior towards the top. Wild-type (WT; A,D), *Shh*−/− (B,E) and *Gli3*−/− (C,F) limbs are shown. Normal expression of *Sall1* and *Sall3* is restricted to the distal-posterior mesenchyme (A,D; black arrows). Both *Sall1* and *Sall3* are downregulated in *Shh*−/− limbs (B,E; red arrows), and are ectopically expressed in the anterior mesenchyme in the *Gli3*−/− limbs (C,F; blue arrows).

the control limb at E11.5 (Fig. 3G,H), which is associated with a smaller autopod area. Given that digit1 develops in the absence of *Shh* (Chiang et al., 2001; Kraus et al., 2001), these results suggest that, in addition to reduced Shh signaling, other mechanisms also contribute to the *Sall1*−/−;*Sall3*−/− limb phenotype.

Relationship between *Sall4-Tbx5* and *Sall1-Sall3*

A recent report using a *Sall4*-gene trap line that would generate a truncated Sall4 protein, similar to the truncated SALL1 in individuals with TBS, suggested that a genetic interaction between *Sall4* and *Tbx5* regulates the development of digit1 (Koshiba-Takeuchi et al., 2006). As digit1 is also affected in the *Sall1*−/−;*Sall3*−/− autopod, this raised the possibility that the phenotype observed in the *Sall1*−/−;*Sall3*−/− autopod could be due to the altered expression of *Sall4*, *Tbx5* (or *Tbx4*). However, we did not observe a significant alteration in the expression of these genes in the *Sall1*−/−;*Sall3*−/− limb bud (see Fig. S2 in the supplementary material). These results indicate that *Sall1* and *Sall3* do not regulate the expression of *Sall4*, *Tbx5* and *Tbx4*.

Conversely, we examined the possibility that *Sall1* and *Sall3* act downstream of *Tbx5*, similar to the case of *Sall4* in the forelimb bud (Harvey and Logan, 2006; Koshiba-Takeuchi et al., 2006). Although a clear downregulation of *Sall4* is reported in *Tbx5*+/− limb buds, we did not observe a significant alteration of *Sall1* and *Sall3* expression in the limb buds between *Tbx5*+/− and wild-type littermates at E11.0 and E11.5 (see Fig. S3 in the supplementary material; data not shown). These results indicate that the expression of *Sall1* and *Sall3* is not regulated by *Tbx5* function. As it has been recently demonstrated that *Tbx5* is required for forelimb initiation, but not for skeletal patterning (Hasson et al., 2007), our data collectively suggest that anterior autopod defects in the *Sall1*−/−;*Sall3*−/− limb are not directly linked to the function of the *Tbx5-Sall4* interaction.

Normal expression of region-specific Hox genes in the absence of *Sall1* and *Sall3*

Studies in invertebrates have suggested that the function of *spalt* is closely associated with that of Hox genes in several developmental contexts (Copf et al., 2006; Galant et al., 2002; Toker et al., 2003). In vertebrates, Hox genes play crucial roles during limb development (reviewed by Zakany and Duboule, 2007). Specifically, *Hoxa13* and *Hoxd13* are required for proper autopod development in mice (Fromental-Ramain et al., 1996). Other Hox
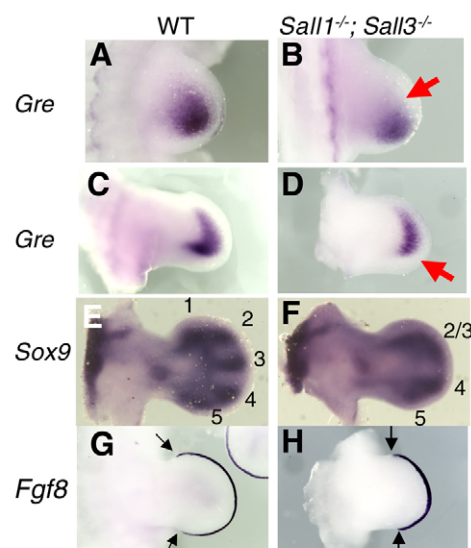
**Fig. 3. Reduced Shh signaling in *Sall1*−/−;*Sall3*−/− mutant limbs.** Dorsal views of E10.5 (A,B) and E11.5 (C-H) limb buds stained with *Grem1* (A-D), *Sox9* (E,F) and *Fgf8* (G,H), with the anterior towards the top. Wild-type (WT; A,C,E,G) and *Sall1*−/−;*Sall3*−/− (B,D,F,H) limbs are shown. (**A-D**) *Grem1* expression is downregulated in the mutant limb (B,D; arrows), compared with control limbs (A,C). (**E,F**) Morphological alteration was visible at E11.5 by *Sox9* in situ hybridization. The control limb has primordia for digit1-digit5 (E). The mutant limb lacks digit1 primordia, exhibits fused digit2 and digit3 primordia, and has delayed separation of digit4 and digit5 primordia (F). (**G,H**) The *Fgf8* expression domain is shorter along the anterior-posterior axis in the AER in the mutant (H), compared with the control limb (G). The anterior and posterior margins of *Fgf8* expression domain are indicated by arrows.

genes also cooperate with these *Hox13* paralogous genes (Kmita et al., 2002; Tarchini et al., 2006). Thus, it is possible that altered Hox expression may account for the *Sall1*−/−;*Sall3*−/− limb phenotype. To examine this possibility, we analyzed the expression of Hoxa and Hoxd genes, which are known to be important for the development of the autopod. We observed similar expression of *Hoxa11*, *Hoxa13*, *Hoxd11*, *Hoxd12* and *Hoxd13* in the control and the *Sall1*−/−;*Sall3*−/− limbs (see Fig. S4 in the supplementary material). Slightly smaller expression domains of *Hoxa13*, *Hoxd12* and *Hoxd13* were detectable in *Sall1*−/−;*Sall3*−/− mutant limbs. However, as morphological alterations are visible at E11.5 (Fig. 3E,F), the minimal changes observed in Hox gene expression are likely to be the consequence, but not the cause, of the morphological alterations. These results indicate that abrogating Sall activity does not affect the regulation of 5′ Hoxa and Hoxd genes during autopod development.

Expression of the Hox target *Epha3* and *Epha4* is altered in the absence of *Sall1* and *Sall3*

Although the expression pattern of Hox genes does not change in the *Sall1*−/−;*Sall3*−/− mutant limbs, it remains possible that the function of Hox proteins is altered. To examine this possibility, we first sought to identify an in vivo readout of Hox activity. A previous comprehensive study has identified several genes regulated by *Hoxd13* (Cobb and Duboule, 2005). *Epha3* is one of the genes characterized as a downstream target of *Hoxd13*. It is not known, however, whether *Epha3* expression is also regulated by *Hoxa13*. We demonstrate, by in situ hybridization analysis, that *Epha3* expression is altered in both *Hoxa13*−/− and *Hoxd13*−/− mutant limbs (Fig. 4A-C).
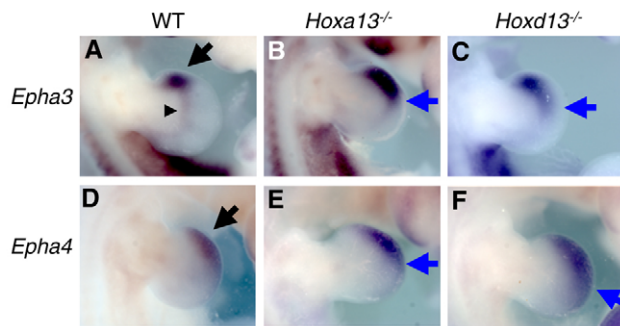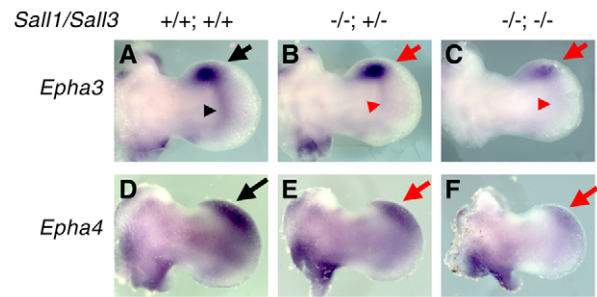
Fig. 4. Expression of *Epha3* and *Epha4* is upregulated in the *Hoxa13* and *Hoxd13* mutants. Dorsal views of E11.5 forelimbs stained with *Epha3* (**A-C**) and *Epha4* (**D-F**) with the anterior towards the top. Wild-type (WT; A,D), *Hoxa13*^{−/−} (B,E) and *Hoxd13*^{−/−} (C,F) limbs are shown. Normal *Epha3* expression in the anterior edge (arrow) and prospective wrist region (arrowhead) (A) is upregulated and expanded posteriorly in the *Hoxa13*^{−/−} (B, arrow) and *Hoxd13*^{−/−} (C, arrow) limbs. Normal *Epha4* expression in the distal-anterior mesenchyme (D, arrow) is upregulated and expanded distal-posteriorly in the *Hoxa13*^{−/−} (E, arrow) and *Hoxd13*^{−/−} (F, arrow) limbs.

Fig. 5. Expression of *Epha3* and *Epha4* is downregulated in *Sall1;Sall3* mutant limbs. Dorsal views of E11.5 forelimbs stained with *Epha3* (A-C) and *Epha4* (D-F) with the anterior towards the top. Wild-type (A,D), *Sall1*^{−/−}*;Sall3*^{+/−} (B,E) and *Sall1*^{−/−}*;Sall3*^{−/−} (C,F) limbs are shown. (**A**) Normal expression of *Epha3* is detected in the anterior edge (arrow) and prospective wrist region (arrowhead). (**B**) *Epha3* expression is downregulated in the *Sall1*^{−/−}*;Sall3*^{+/−} limb. (**C**) The anterior edge expression is more severely downregulated and the prospective wrist region expression is undetectable in the *Sall1*^{−/−}*;Sall3*^{−/−} limb. (**D**) Normal *Epha4* expression in detected the distal anterior mesenchyme (arrow). (**E,F**) *Epha4* expression is downregulated in the *Sall1*^{−/−}*;Sall3*^{+/−} limb (E), and is more severely downregulated in the *Sall1*^{−/−}*;Sall3*^{−/−} limb (F).

This change includes not only an upregulation of expression but is also an expansion of the expression domain from the anterior edge to the distal middle region. Furthermore, we found that *Epha4* was also mis-expressed in *Hox13* mutants, making this gene a likely Hox gene target. Similar to the case of *Epha3*, *Epha4* expression is upregulated in both *Hoxa13*^{−/−} and *Hoxd13*^{−/−} mutant limbs. These results indicate that *Hoxa13* and *Hoxd13* repress *Epha3* and *Epha4* expression, and that the expression of *Epha3* and *Epha4* is a bona fide indicator of Hoxa13 and Hoxd13 activity in the limb bud.

In order to examine the possibility that Hox gene function is altered in the absence of *Sall1* and *Sall3*, we analyzed the expression of the Hox target genes *Epha3* and *Epha4* in the *Sall* mutant limbs. In this analysis, we compared controls with *Sall1*^{−/−}*;Sall3*^{+/−} and *Sall1*^{−/−}*;Sall3*^{−/−} mutant limbs in order to clarify whether elimination of more Sall gene alleles has a more severe effect on the expression of Hox targets, as we showed above for limb skeletal elements. In situ hybridization of *Epha3* and *Epha4* demonstrated that the extent of mis-expression was directly correlated with Sall gene dose (Fig. 5). In the *Sall1*^{−/−}*;Sall3*^{+/−} mutant limb, expression of *Epha3* and *Epha4* is slightly, but clearly, reduced; expression of both genes in the prospective carpal element-forming region is downregulated, and anterior expression of *Epha4* is also downregulated (Fig. 5B,E). In *Sall1*^{−/−}*;Sall3*^{−/−} mutant limbs, the expression of *Epha3* and *Epha4* is more significantly reduced, and the anterior mesenchyme expression of both genes is severely downregulated (Fig. 5C,F). Our results also suggest that *Sall1* and *Sall3* regulate expression of *Epha3* and *Epha4*. As *Hoxa13* and *Hoxd13* repress expression of *Epha3* and *Epha4*, these results suggest a possible gain of Hox gene function in *Sall1;Sall3* mutant limbs.

Hox represses Sall expression

Our results suggest a relationship between Hox activity and Sall activity. We hypothesized that Hox activity represses the expression of *Sall1* and *Sall3*, resulting in downregulation of *Epha3* and *Epha4* expression. To test this possibility, we analyzed the expression of *Sall1* and *Sall3* in *Hox* mutants. Normal expression of *Sall1* and *Sall3* starts to regress from the most distal mesenchyme in the E11.5

hindlimb (Fig. 6A,E) (Buck et al., 2001; Ott et al., 2001). Expression of *Sall1* and *Sall3* in the *Hoxa13*^{−/−} mutant limb is slightly stronger than that of a wild-type E11.5 littermate hindlimbs (Fig. 6B,F). In the *Hoxd13*^{−/−} mutant limb, the expression of *Sall1* and *Sall3* is upregulated, and the expression was prolonged in the most distal region when compared with a wild-type littermate (Fig. 6C,G). In the *Hoxa13*^{−/−}*;Hoxd13*^{−/−} mutant limb, the expression of *Sall1* and *Sall3* is stronger and more expanded in the large region of the distal mesenchyme when compared with single *Hoxa13* or *Hoxd13* mutant limbs (Fig. 6D,H). These results indicate a synergistic activity of Hoxa13 and Hoxd13 in repressing *Sall1* and *Sall3* expression.

Sall and Hox compete for a target sequence

As *Hox* expression is not affected in the absence of *Sall1* and *Sall3* (see Fig. S4 in the supplementary material), the possible gain of *Hox* function in *Sall1*^{−/−}*;Sall3*^{−/−} limbs might be by post-transcriptional regulation. A possible mechanism for such regulation could be that Sall and Hox compete for regulatory elements of common target genes such as *Epha3* and *Epha4*. In an effort to address this, we found that the mouse *Epha4* gene has an AT-rich stretch in the upstream region. At –2028 bp from the transcription start position, we found two recently identified, tandemly positioned, AT-rich Sall1 consensus sequences (Lauberth et al., 2007; Yamashita et al., 2007).

As Hox proteins have preferential binding to AT-rich sequences (Pearson et al., 2005), these proteins may act antagonistically in the upstream region for the transcriptional regulation of *Epha4*. Therefore, we analyzed whether Sall1, Hoxa13 and Hoxd13 can recognize the AT-rich Sall1 consensus sequence upstream of the *Epha4* gene by EMSA. As shown in Fig. 7A, in vitro translated HA-tagged Sall1 binds to the wild-type probe (arrow). The specificity is confirmed by the supershift induced by the anti-HA antibody (asterisk). With a probe carrying two types of mutations in distinct domains, the binding clearly became weaker. The binding was more affected by introducing two-point mutations on the 3′ side (M1 probe) than four-point mutations on the 5′ side (M2 probe). With a probe containing multiple mutations that disrupted the AT-rich
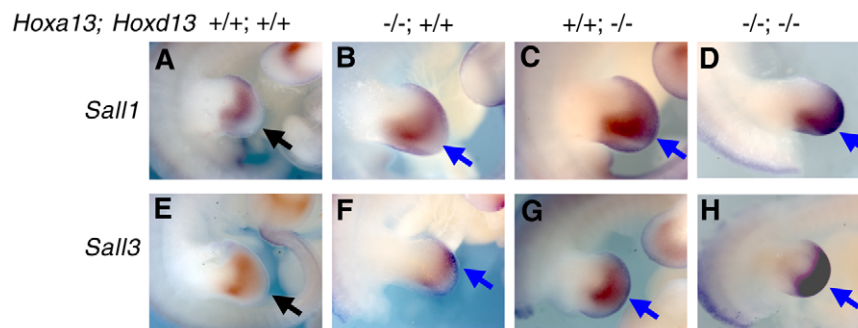
Fig. 6. *Hox* represses the expression of *Sall1* and *Sall3*. Dorsal views of E11.5 hindlimbs stained with *Sall1* (A-D) and *Sall3* (E-H) with anterior towards the top. Wild-type (A,E), *Hoxa13*$^{-/-}$ (B,F), *Hoxd13*$^{-/-}$ (C,G) and *Hoxa13*$^{-/-}$;*Hoxd13*$^{-/-}$ (D,H) limbs are shown. (**A**) Normal *Sall1* expression starts to regress from the most distal mesenchyme (arrow). (**B**) In the *Hoxa13*$^{-/-}$ limb, the *Sall1* expression domain became larger and the signal stronger. (**C**) In the *Hoxd13*$^{-/-}$ limb, the *Sall1* signal is detected in the distal region (arrow) and is stronger than that in the wild type. (**D**) In the *Hoxa13*$^{-/-}$;*Hoxd13*$^{-/-}$ limb, a large domain in the distal mesenchyme expresses significantly high levels of *Sall1* (arrow). (**E**) Normal *Sall3* expression also starts to regress from the most distal mesenchyme (arrow). (**F**) In the *Hoxa13*$^{-/-}$ limb, higher level of *Sall3* expression is detected in the anterior mesenchyme (arrow). (**G**) In the *Hoxd13*$^{-/-}$ limb, higher level of *Sall3* expression is detected in the distal-middle region (arrow). (**H**) In the *Hoxa13*$^{-/-}$;*Hoxd13*$^{-/-}$ limb, strong expression of *Sall3* is detected in the wide region of the distal mesenchyme.

sequence (M3 probe), the binding was completely abolished. Conversely, when these wild-type and mutant probes are used as excess amount of cold competitors, we observed complementary results. These results demonstrate that Sall1 binds to the AT-rich sequence in the upstream region of the *Epha4* gene.

Next, we performed similar experiments with in vitro translated Flag-tagged Hoxa13 and Flag-tagged Hoxd13 (Fig. 7B,C). Both Hoxa13 and Hoxd13 bound the wild-type probe (arrows), and the specificity was confirmed by the supershift induced by the anti-Flag antibody (asterisks). The M1 mutant probe showed reduced binding, although the M2 mutant probe had little effect on binding. Introducing multiple mutations (M3) abolished binding. A complementary result was also observed by using these probes as cold competitors. These results demonstrate that Hoxa13 and Hoxd13 also recognize the upstream sequence of the *Epha4* gene that is recognized by Sall1.

The results obtained from DNA-binding assays suggest that the competition for a common binding sequence could be one of the mechanisms for the antagonistic function between Sall and Hox. We tested this possibility by examining the relationship between Sall1 and Hox13 for a common binding sequence in vitro. Sall1, Hoxa13 and Hoxd13 bind to the wild-type probe (Fig. 7A-C), and when Sall1 was present together with Hox13, the binding of Hox13 to the probe was reduced (Fig. 7D). The Sall1-DNA complex also became weaker. This suggests that Sall1 and Hoxa13 (or Hoxd13) compete for the target sequence and that such a mechanism could contribute to the mutual antagonistic function between Sall and Hox proteins.

We further examined whether such a competition could functionally contribute to the regulation of Hox activity. For this purpose, we set up a luciferase reporter assay by using an *Epha4* upstream region that contains the Sall-Hox binding site. *Hoxd13* activated reporter activity, whereas *Hoxa13* and *Sall1* did not activate this element. Importantly, co-expression of *Sall1* significantly reduced *Hoxd13*-dependent reporter activation. Although *Hoxa13* and *Hoxd13* show different functional contributions to this specific upstream element, similar to the autopod development in vivo (Fromental-Ramain et al., 1996), our data support the idea that DNA binding competition could contribute to the functional antagonism between Sall and Hox13.

DISCUSSION
Sall genes regulate autopod development
Most of the human TBS defects seem to involve the dominant-negative action of a truncated SALL1 protein. Indeed, defects in the anterior part of hands and feet, renal agenesis and anal deformities were observed in a mutant mouse line carrying a truncated *Sall1* form that can interact with all Sall proteins (Kiefer et al., 2003). The absence of limb phenotypes in mice mutant for individual *Sall* gene is most likely due to functional redundancy between the different members of the *Sall* gene family. The autopod phenotype of *Sall1*$^{-/-}$;*Sall3*$^{-/-}$ mutant confirmed that functional redundancy exists between *Sall1* and *Sall3*, though their functional activity is not equivalent. Based on the skeletal phenotypes of the *Sall1;Sall3* allelic series, *Sall1* appears to have a more important contribution than does *Sall3*. Indeed, *Sall1*$^{-/-}$;*Sall3*$^{-/-}$ mutants showed the strongest phenotype, and milder phenotypes were apparent with the addition of *Sall3* functional alleles (*Sall1*$^{-/-}$;*Sall3*$^{+/+}$ mutants exhibit a milder phenotype than do *Sall1*$^{-/-}$;*Sall3*$^{+/-}$ mutants). No limb alterations were observed in mice with other genotypes such as *Sall1*$^{+/-}$;*Sall3*$^{-/-}$, *Sall1*$^{+/-}$;*Sall3*$^{+/-}$ (Fig. 1). Given that mice expressing truncated *Sall1* exhibit loss of digit1 and several carpal elements (Kiefer et al., 2003), our data indicate that the dominant-negative action of a truncated Sall1 might inhibit not only Sall1 but also Sall3 function. Overall, the findings described here support the concept that *Sall1* and *Sall3* have partially redundant activity. Such redundant activity among members of the *Sall* gene family is also observed in *Drosophila*, where loss of *spalt* and *spalt-related* genes cause defects in multiple organs (Dong et al., 2003). Thus, the idea that Sall genes function cooperatively in organogenesis seems to be a conserved feature in vertebrates and invertebrates.

Mouse genetic studies shown here, as well as previous studies by others, suggest an organ-specific requirement for different SALL genes in relation to the TBS. The kidney agenesis appears to be caused mainly by a loss of *Sall1* (Nishinakamura et al., 2001), the anal and heart phenotypes are probably due to inhibition of both *Sall4* and *Sall1* function (Sakaki-Yumoto et al., 2006), and the limb phenotype seems to be caused by a reduction of *Sall1* and *Sall3* function (this study). *Sall2* appears to be dispensable for limb development, as the *Sall1*$^{-/-}$;*Sall2*$^{-/-}$;*Sall3*$^{-/-}$ triple mutant limb was indistinguishable from that of *Sall1*$^{-/-}$;*Sall3*$^{-/-}$ mutant limb (data not
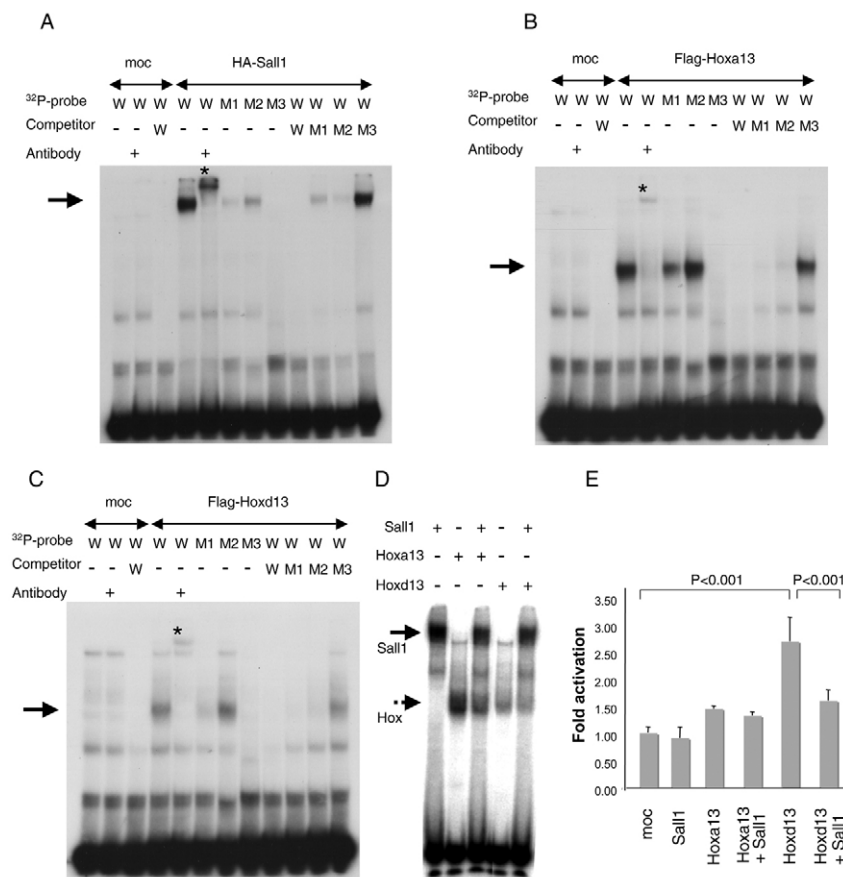
Fig. 7. Sall1 modulates Hox activity post-transcriptionally. (**A**) EMSA assay with the HA-Sall1 protein. Sall1 recognizes the *Epha4* upstream element (arrow). The specificity was confirmed by supershift induced by anti-HA antibody (asterisk). The Sall1-probe complex became weaker by introducing mutations in the probe (M1, M2), and is abolished by introducing multiple mutations (M3). (**B,C**) EMSA assay with Flag-Hoxa13 protein (B) and Flag-Hoxd13 protein (C). Both Hoxa13 and Hoxd13 recognize the *Epha4* upstream element (arrows). The specificity was confirmed by supershift induced by anti-Flag antibody (asterisks). The Hox13-probe complex was weaker with the M1 mutant probe, but was not severely affected with the M2 mutant probe. The binding was abolished with the M3 mutant probe containing multiple mutations. M1, mutant1; M2, mutant2; M3, mutant3 (see Materials and methods). (**D**) Sall1 and Hox13 compete for an *Epha4* upstream element. Specific bands formed between ^{32}P-labeled wild-type probe and Sall1 (arrow), and between ^{32}P-labeled wild-type probe and Hox13 (broken arrow) were detected. By co-incubating with Sall1, the Hoxa13-DNA complex and the Hoxd13-DNA complex became weaker. (**E**) Luciferase-reporter assay showing Hox-activity modulation by Sall1. The reporter construct was co-transfected with 100 ng of Hoxa13, Hoxd13 and/or Sall1 expression constructs, together with 20 ng pRL-TK. Data are shown as mean±s.d. Significant differences between mock transfected (moc), and Hoxd13, Hoxd13 and Hoxd13+Sall1 are detected ($P<0.001$).

shown). As *Sall4* is also expressed in the limb mesenchyme, it is possible that *Sall4* acts together with *Sall1* and *Sall3*. As *Sall4*$^{-/-}$ embryos die soon after implantation (Elling et al., 2006; Sakaki-Yumoto et al., 2006; Warren et al., 2007), the generation of a *Sall4* conditional allele is necessary to investigate this issue.

Relationship between Sall4 and Sall1-Sall3

A recent report with a *Sall4* gene trap line suggests that *Sall4* is involved in anterior autopod patterning through genetic interaction with *Tbx5* (Koshiba-Takeuchi et al., 2006). Our analyses suggest that the expression of *Sall1* and *Sall3* is not regulated by *Tbx5*, and that the *Sall1*$^{-/-}$;*Sall3*$^{-/-}$ autopod phenotype is unlikely to be linked to *Sall4* and *Tbx5* (see Fig. S2 in the supplementary material). The *Sall4* gene trap allele would generate a truncated form of Sall4 that might act as a dominant negative, similar to the truncated SALL1 in individuals with TBS. Thus, the observed phenotype in the *Sall4*$^{GT/+}$;*Tbx5*$^{+/-}$ limb might involve reduced activity of Sall1 and Sall3 by a dominant-negative action of the truncated Sall4, in addition to *Sall4* haploinsufficiency. Further investigation of the *Sall4* loss-of-function phenotype, alone and in combination with *Sall1* and *Sall3*, will be of particular interest for a comprehensive analysis of the function and contribution of the Sall gene family during limb development.

Sall and Shh signaling

Sall activity appears to be part of Shh pathway. Both *Shh* and *Gli3* signaling have an impact on *Sall1* and *Sall3* expression (Fig. 2). Experiments in chicks have demonstrated that *Sall1* expression in limb buds is regulated by Shh and Fgf, which suggested a possible

involvement of *Sall1* in distal limb bud patterning (Farrell and Munsterberg, 2000). The reduced expression of *Grem1*, a Shh-signaling target gene in distal/posterior mesenchyme suggest that Sall function acts to maintain proper levels of Shh signaling in the limb mesenchyme (Fig. 3). Abrogating *Shh* function at various time points during limb development revealed that digit3 formation is the most sensitive to the loss of *Shh* (Scherz et al., 2007; Zhu et al., 2008). Interestingly, the *Sall1;Sall3* mutation affects primarily the formation of digit3 (Fig. 1). Such similarity further supports the idea that Sall1-Sall3 contribution to Shh signaling. However, the fact that digit5, a second digit sensitive to the loss of Shh activity, developed in the *Sall1*$^{-/-}$;*Sall3*$^{-/-}$ mutant limb suggests that Sall1-Sall3 activity is not a major player in Shh signaling events. Our observation that *Grem1* expression is not abolished but is partially downregulated (Fig. 3) also supports this idea. It is conceivable that *Sall4*, which is expressed in the distal mesenchyme of the developing limb (Kohlhase et al., 2002), partially compensates for the loss of *Sall1* and *Sall3*. Besides a possible redundancy between *Sall1*, *Sall3* and *Sall4*, the loss of digit1, a Shh-independent digit, in the *Sall1*$^{-/-}$;*Sall3*$^{-/-}$ mutant suggests that *Sall1*$^{-/-}$;*Sall3*$^{-/-}$ phenotype is not exclusively due to reduced Shh signaling.

Epha3 and *Epha4* as targets of Hox activity

Genetic lineage tracing experiments have demonstrated that digit1, which is missing in the *Sall1*$^{-/-}$;*Sall3*$^{-/-}$ mutant autopod, is developed independently of Shh activity (Ahn and Joyner, 2004; Harfe et al., 2004). Furthermore, digit1 develops in the absence of *Shh* function (Chiang et al., 2001; Kraus et al., 2001). Thus, the loss of digit 1 in the *Sall1*$^{-/-}$;*Sall3*$^{-/-}$ mutant limb most probably involves

a Shh-independent process. Several lines of evidence led us to examine the possible role of Hox genes in this phenotype: (1) in invertebrates, the function of *spalt* gene has been closely associated with the functions of Hox genes in several developmental contexts (Copf et al., 2006); (2) mice with compound mutations in the 5′ Hoxd genes, such as *Hoxd11*, *Hoxd12* and *Hoxd13*, show defects in carpal and metacarpal elements (Davis and Capecchi, 1996), which are also observed in the *Sall1⁻/⁻;Sall3⁻/⁻* limb; (3) mice with compound mutations in *Hoxa13* and *Hoxd13* exhibit complex autopod phenotypes, such as abnormal condensation and fusion of cartilage elements, demonstrating that correct levels of *Hoxa13* and *Hoxd13* function is important for autopod development (Fromental-Ramain et al., 1996); (4) a recent analysis demonstrated that differences in the local level of Hox transcripts specifically regulates digit1 morphogenesis (Montavon et al., 2008). Thus, given that Sall genes encode transcription factors, they might be part of the mechanisms that control Hox gene expression in limbs, which would explain, at least in part, the Sall mutant phenotype. However, we did not observe alteration in the expression of 5′ Hox genes in Sall1/Sall3 mutants (see Fig. S4 in the supplementary material). An alternative possibility is that Sall proteins have an impact on Hox function at a post-transcriptional level. Recent analyses have identified several genes regulated by 5′ Hoxd genes (Cobb and Duboule, 2005). One such gene is *Epha3*, the expression of which is negatively regulated by 5′ Hoxd genes. Our genetic analyses further uncovered that *Epha4* is also regulated by *Hoxa13* and *Hoxd13* (Fig. 4). As such, *Epha3* and *Epha4* can be used as bona fide indicators of Hoxa13 and Hoxd13 activity. Even though Hox mutant phenotypes appear to involve complex processes and numerous target genes, analysis of *Epha3* and *Epha4* expression allowed us to evaluate the activities of Hoxa13 and Hoxd13, and revealed that Hox13 and Sall proteins compete for binding on common target sequences.

It is not completely understood how Hox regulates limb morphogenesis. Studies have suggested that *Hoxa13* regulates cell surface affinity, which affects region-specific cell-cell aggregation and segregation (Stadler et al., 2001; Yokouchi et al., 1995). In these studies, it is suggested that Hoxa13-mediated boundary formation may be an important process for morphogenesis of cartilage elements, and further suggested that the Eph-ephrin system might be involved in the regulation of cell surface affinity and morphogenesis. Eph encodes a receptor tyrosine kinase and ephrin encodes a transmembrane or glycosylphosphatidylinositol-anchored membrane protein. Their interaction is known to regulate cell-cell repulsion as well as attraction, and discrete spatial expression of Ephs and ephrins is known to be important for boundary formation during tissue morphogenesis (Holder and Klein, 1999; Klein, 2004; Poliakov et al., 2004). Interestingly, ectopic expression of ephrin A2 in the developing chick limb, which caused the formation of abnormal ephrin A2 expression boundaries, resulted in abnormal chondrogenic progenitor segregation, leading to a disruption of cartilage morphology (Wada et al., 2003). Furthermore, inactivation of ephrin B1, which causes mosaic expression of the X-linked ephrin B1 in heterozygous mice, generated ectopic ephrin B1-EphB interactions and abnormal digit formation (Compagni et al., 2003). These studies link Hox activity and cell-cell interaction in the control of skeletal elements formation. Hox genes regulate the Eph-ephrin system in other organs (Bruhl et al., 2004; Shaut et al., 2007) and might be important during development of other organs. Thus, studying compound mutants with Eph and ephrin genes in the future could contribute to understanding the role of cell-cell interaction for cartilage morphogenesis.

Sall modulates Hox activity in the limb

Our analysis suggests that *Sall* and *Hox* have antagonistic functions during autopod development. By using the expression of *Epha3* and *Epha4* as a marker of *Hox* function, we have found that Hox and Sall have an opposite impact on their expression. Furthermore, our genetic analysis clearly demonstrates that *Hoxa13* and *Hoxd13* repress expression of *Sall1* and *Sall3* in the autopod. In turn, Sall proteins antagonize Hox function at a post-transcriptional level. Our EMSA assays demonstrated that Sall1, Hoxa13 and Hoxd13 bind to a sequence upstream of the *Epha4* gene, suggesting that they might directly regulate *Epha4* expression. Moreover, when co-incubated together, Sall1 competes with Hox13 for binding on the target DNA sequence (Fig. 7D). Luciferase-reporter assay experiments further supported that such competition could contribute to modulating transcriptional activity (Fig. 7E). *Hoxd13* activated the reporter with the *Epha4*-upstream element, whereas genetic evidence has demonstrated *Hoxd13* as a repressor. Such a context-dependent activator/repressor conversion has been known to occur with several transcription factors, including Hox (Fry and Farnham, 1999; Svingen and Tonissen, 2006). Importantly, co-expressed *Sall1* repressed *Hoxd13*-dependent reporter activation, whereas *Sall1* alone did not show an effect on this reporter (Fig. 7E). The reason that *Hoxa13* did not show significant activation of this reporter is unclear. As the expression of *Epha4* is more affected in the *Hoxd13⁻/⁻* limb than in the *Hoxa13⁻/⁻* limb (Fig. 4), the contribution of *Hoxd13* to the regulation of *Epha4* expression might be more significant than that by *Hoxa13*, and reporter activation in vitro might reflect such a difference. Alternatively, such a reporter assay might not completely recapitulate in vivo functions. Nonetheless, our data demonstrate that Sall and Hox can compete for a common target sequence and such competition could contribute to functional modulation. Such competition might, at least in part, contribute to their possible antagonistic function.

Target recognition by Hox proteins is not very strict, favoring a four-base AT-rich core sequence (Pearson et al., 2005). Therefore, depending on the molecular partners that might affect stringency and affinity to target sequences (Svingen and Tonissen, 2006), Hox proteins might bind to a variety of regulatory elements. Contrary to Hox, target recognition by Sall1 is rather stringent (Lauberth et al., 2007; Yamashita et al., 2007). Thus, antagonism by Sall proteins might serve to add local and developmentally timed specific modulation of Hox activity in the autopod. In turn, such antagonistic interactions between Hox and Sall in the autopod might contribute to fine-tuning local cell-cell affinity, leading to segregation or aggregation of chondrogenic progenitors, and thus contribute to generating the complex cartilage architecture of the vertebrate autopod.

References
Ahn, S. and Joyner, A. L. (2004). Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. *Cell* **118**, 505-516.

Borozdin, W., Steinmann, K., Albrecht, B., Bottani, A., Devriendt, K., Leipoldt, M. and Kohlhase, J. (2006). Detection of heterozygous SALL1 deletions by quantitative real time PCR proves the contribution of a SALL1 dosage effect in the pathogenesis of Townes-Brocks syndrome. *Hum. Mutat.* **27**, 211-212.

Bruhl, T., Urbich, C., Aicher, D., Acker-Palmer, A., Zeiher, A. M. and Dimmeler, S. (2004). Homeobox A9 transcriptionally regulates the EphB4 receptor to modulate endothelial cell migration and tube formation. *Circ. Res.* **94**, 743-751.

Bruneau, B. G., Nemer, G., Schmitt, J. P., Charron, F., Robitaille, L., Caron, S., Conner, D. A., Gessler, M., Nemer, M., Seidman, C. E. et al. (2001). A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* **106**, 709-721.

Buck, A., Kispert, A. and Kohlhase, J. (2001). Embryonic expression of the murine homologue of SALL1, the gene mutated in Townes-Brocks syndrome. *Mech. Dev.* **104**, 143-146.

Buscher, D., Grotewold, L. and Ruther, U. (1998). The XtJ allele generates a Gli3 fusion transcript. *Mamm. Genome* **9**, 676-678.

Capdevila, J. and Izpisua Belmonte, J. C. (2001). Patterning mechanisms controlling vertebrate limb development. *Annu. Rev. Cell Dev. Biol.* **17**, 87-132.

Capdevila, J., Tsukui, T., Rodriguez Esteban, C., Zappavigna, V. and Izpisua Belmonte, J. C. (1999). Control of vertebrate limb outgrowth by the proximal factor Meis2 and distal antagonism of BMPs by Gremlin. *Mol. Cell* **4**, 839-849.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.

Chiang, C., Litingtung, Y., Harris, M. P., Simandl, B. K., Li, Y., Beachy, P. A. and Fallon, J. F. (2001). Manifestation of the limb prepattern: limb development in the absence of sonic hedgehog function. *Dev. Biol.* **236**, 421-435.

Cobb, J. and Duboule, D. (2005). Comparative analysis of genes downstream of the Hoxd cluster in developing digits and external genitalia. *Development* **132**, 3055-3067.

Compagni, A., Logan, M., Klein, R. and Adams, R. H. (2003). Control of skeletal patterning by ephrinB1-EphB interactions. *Dev. Cell* **5**, 217-230.

Copf, T., Rabet, N. and Averof, M. (2006). Knockdown of spalt function by RNAi causes de-repression of Hox genes and homeotic transformations in the crustacean Artemia franciscana. *Dev. Biol.* **298**, 87-94.

Davis, A. P. and Capecchi, M. R. (1996). A mutational analysis of the 5′ HoxD genes: dissection of genetic interactions during limb development in the mouse. *Development* **122**, 1175-1185.

Dong, P. D., Todi, S. V., Eberl, D. F. and Boekhoff-Falk, G. (2003). Drosophila spalt/spalt-related mutants exhibit Townes-Brocks' syndrome phenotypes. *Proc. Natl. Acad. Sci. USA* **100**, 10293-10298.

Dudley, A. T., Ros, M. A. and Tabin, C. J. (2002). A re-examination of proximodistal patterning during vertebrate limb development. *Nature* **418**, 539-544.

Elling, U., Klasen, C., Eisenberger, T., Anlag, K. and Treier, M. (2006). Murine inner cell mass-derived lineages depend on Sall4 function. *Proc. Natl. Acad. Sci. USA* **103**, 16319-16324.

Farrell, E. R. and Munsterberg, A. E. (2000). csal1 is controlled by a combination of FGF and Wnt signals in developing limb buds. *Dev. Biol.* **225**, 447-458.

Fromental-Ramain, C., Warot, X., Messadecq, N., LeMeur, M., Dolle, P. and Chambon, P. (1996). Hoxa-13 and Hoxd-13 play a crucial role in the patterning of the limb autopod. *Development* **122**, 2997-3011.

Fry, C. J. and Farnham, P. J. (1999). Context-dependent transcriptional regulation. *J. Biol. Chem.* **274**, 29583-29586.

Galant, R., Walsh, C. M. and Carroll, S. B. (2002). Hox repression of a target gene: extradenticle-independent, additive action through multiple monomer binding sites. *Development* **129**, 3115-3126.

Goff, D. J. and Tabin, C. J. (1997). Analysis of Hoxd-13 and Hoxd-11 misexpression in chick limb buds reveals that Hox genes affect both bone condensation and growth. *Development* **124**, 627-636.

Goodman, F. R. (2002). Limb malformations and the human HOX genes. *Am. J. Med. Genet.* **112**, 256-265.

Grant, K., Hanna-Rose, W. and Han, M. (2000). sem-4 promotes vulval cell-fate determination in Caenorhabditis elegans through regulation of lin-39 Hox. *Dev. Biol.* **224**, 496-506.

Harfe, B. D., Scherz, P. J., Nissim, S., Tian, H., McMahon, A. P. and Tabin, C. J. (2004). Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell* **118**, 517-528.

Harvey, S. A. and Logan, M. P. (2006). sall4 acts downstream of tbx5 and is required for pectoral fin outgrowth. *Development* **133**, 1165-1173.

Hasson, P., Del Buono, J. and Logan, M. P. (2007). Tbx5 is dispensable for forelimb outgrowth. *Development* **134**, 85-92.

Holder, N. and Klein, R. (1999). Eph receptors and ephrins: effectors of morphogenesis. *Development* **126**, 2033-2044.

Kawakami, Y., Capdevila, J., Buscher, D., Itoh, T., Rodriguez Esteban, C. and Izpisua Belmonte, J. C. (2001). WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. *Cell* **104**, 891-900.

Khokha, M. K., Hsu, D., Brunet, L. J., Dionne, M. S. and Harland, R. M. (2003). Gremlin is the BMP antagonist required for maintenance of Shh and Fgf signals during limb patterning. *Nat. Genet.* **34**, 303-307.

Kiefer, S. M., Ohlemiller, K. K., Yang, J., McDill, B. W., Kohlhase, J. and Rauchman, M. (2003). Expression of a truncated Sall1 transcriptional repressor is responsible for Townes-Brocks syndrome birth defects. *Hum. Mol. Genet.* **12**, 2221-2227.

Kiefer, S. M., Robbins, L., Barina, A., Zhang, Z. and Rauchman, M. (2008). SALL1 truncated protein expression in Townes-Brocks syndrome leads to ectopic expression of downstream genes. *Hum. Mutat.* **29**, 1133-1140.

Klein, R. (2004). Eph/ephrin signaling in morphogenesis, neural development and plasticity. *Curr. Opin. Cell Biol.* **16**, 580-589.

Kmita, M., Kondo, T. and Duboule, D. (2000). Targeted inversion of a polar silencer within the HoxD complex re-allocates domains of enhancer sharing. *Nat. Genet.* **26**, 451-454.

Kmita, M., Fraudeau, N., Herault, Y. and Duboule, D. (2002). Serial deletions and duplications suggest a mechanism for the collinearity of Hoxd genes in limbs. *Nature* **420**, 145-150.

Kmita, M., Tarchini, B., Zakany, J., Logan, M., Tabin, C. J. and Duboule, D. (2005). Early developmental arrest of mammalian limbs lacking HoxA/HoxD gene function. *Nature* **435**, 1113-1116.

Kohlhase, J. (2000). SALL1 mutations in Townes-Brocks syndrome and related disorders. *Hum. Mutat.* **16**, 460-466.

Kohlhase, J., Wischermann, A., Reichenbach, H., Froster, U. and Engel, W. (1998). Mutations in the SALL1 putative transcription factor gene cause Townes-Brocks syndrome. *Nat. Genet.* **18**, 81-83.

Kohlhase, J., Heinrich, M., Liebers, M., Frohlich Archangelo, L., Reardon, W. and Kispert, A. (2002). Cloning and expression analysis of SALL4, the murine homologue of the gene mutated in Okihiro syndrome. *Cytogenet. Genome Res.* **98**, 274-277.

Koshiba-Takeuchi, K., Takeuchi, J. K., Arruda, E. P., Kathiriya, I. S., Mo, R., Hui, C. C., Srivastava, D. and Bruneau, B. G. (2006). Cooperative and antagonistic interactions between Sall4 and Tbx5 pattern the mouse limb and heart. *Nat. Genet.* **38**, 175-183.

Kraus, P., Fraidenraich, D. and Loomis, C. A. (2001). Some distal limb structures develop in mice lacking Sonic hedgehog signaling. *Mech. Dev.* **100**, 45-58.

Lappin, T. R., Grier, D. G., Thompson, A. and Halliday, H. L. (2006). HOX genes: seductive science, mysterious mechanisms. *Ulster Med. J.* **75**, 23-31.

Lauberth, S. M., Bilyeu, A. C., Firulli, B. A., Kroll, K. L. and Rauchman, M. (2007). A phosphomimetic mutation in the Sall1 repression motif disrupts recruitment of the nucleosome remodeling and deacetylase complex and repression of Gbx2. *J. Biol. Chem.* **282**, 34858-34868.

Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A. and Tabin, C. (1994). Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79**, 993-1003.

Litingtung, Y., Dahn, R. D., Li, Y., Fallon, J. F. and Chiang, C. (2002). Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. *Nature* **418**, 979-983.

Mariani, F. V., Ahn, C. P. and Martin, G. R. (2008). Genetic evidence that FGFs have an instructive role in limb proximal-distal patterning. *Nature* **453**, 401-405.

McLeod, M. J. (1980). Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. *Teratology* **22**, 299-301.

Merino, R., Rodriguez-Leon, J., Macias, D., Ganan, Y., Economides, A. N. and Hurle, J. M. (1999). The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. *Development* **126**, 5515-5522.

Michos, O., Panman, L., Vintersten, K., Beier, K., Zeller, R. and Zuniga, A. (2004). Gremlin-mediated BMP antagonism induces the epithelial-mesenchymal feedback signaling controlling metanephric kidney and limb organogenesis. *Development* **131**, 3401-3410.

Montavon, T., Le Garrec, J. F., Kerszberg, M. and Duboule, D. (2008). Modeling Hox gene regulation in digits: reverse collinearity and the molecular origin of thumbness. *Genes Dev.* **22**, 346-359.

Nishinakamura, R. and Osafune, K. (2006). Essential roles of Sall family genes in kidney development. *J. Physiol. Sci.* **56**, 131-136.

Nishinakamura, R., Matsumoto, Y., Nakao, K., Nakamura, K., Sato, A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Scully, S., Lacey, D. L. et al. (2001). Murine homolog of SALL1 is essential for ureteric bud invasion in kidney development. *Development* **128**, 3105-3115.

Nissim, S., Hasso, S. M., Fallon, J. F. and Tabin, C. J. (2006). Regulation of Gremlin expression in the posterior limb bud. *Dev. Biol.* **299**, 12-21.

Niswander, L. (2003). Pattern formation: old models out on a limb. *Nat. Rev. Genet.* **4**, 133-143.

Ott, T., Parrish, M., Bond, K., Schwaeger-Nickolenko, A. and Monaghan, A. P. (2001). A new member of the spalt like zinc finger protein family, Msal-3, is expressed in the CNS and sites of epithelial/mesenchymal interaction. *Mech. Dev.* **101**, 203-207.

Panman, L., Galli, A., Lagarde, N., Michos, O., Soete, G., Zuniga, A. and Zeller, R. (2006). Differential regulation of gene expression in the digit forming

area of the mouse limb bud by SHH and gremlin 1/FGF-mediated epithelial-mesenchymal signalling. *Development* **133**, 3419-3428.

Parrish, M., Ott, T., Lance-Jones, C., Schuetz, G., Schwaeger-Nickolenko, A. and Monaghan, A. P. (2004). Loss of the Sall3 gene leads to palate deficiency, abnormalities in cranial nerves, and perinatal lethality. *Mol. Cell. Biol.* **24**, 7102-7112.

Pearson, J. C., Lemons, D. and McGinnis, W. (2005). Modulating Hox gene functions during animal body patterning. *Nat. Rev. Genet.* **6**, 893-904.

Poliakov, A., Cotrina, M. and Wilkinson, D. G. (2004). Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. *Dev. Cell* **7**, 465-480.

Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.

Sakaki-Yumoto, M., Kobayashi, C., Sato, A., Fujimura, S., Matsumoto, Y., Takasato, M., Kodama, T., Aburatani, H., Asashima, M., Yoshida, N. et al. (2006). The murine homolog of SALL4, a causative gene in Okihiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with Sall1 in anorectal, heart, brain and kidney development. *Development* **133**, 3005-3013.

Sato, A., Matsumoto, Y., Koide, U., Kataoka, Y., Yoshida, N., Yokota, T., Asashima, M. and Nishinakamura, R. (2003). Zinc finger protein sall2 is not essential for embryonic and kidney development. *Mol. Cell. Biol.* **23**, 62-69.

Scherz, P. J., Harfe, B. D., McMahon, A. P. and Tabin, C. J. (2004). The limb bud Shh-Fgf feedback loop is terminated by expansion of former ZPA cells. *Science* **305**, 396-399.

Scherz, P. J., McGlinn, E., Nissim, S. and Tabin, C. J. (2007). Extended exposure to Sonic hedgehog is required for patterning the posterior digits of the vertebrate limb. *Dev. Biol.* **308**, 343-354.

Shaut, C. A., Saneyoshi, C., Morgan, E. A., Knosp, W. M., Sexton, D. R. and Stadler, H. S. (2007). HOXA13 directly regulates EphA6 and EphA7 expression in the genital tubercle vascular endothelia. *Dev. Dyn.* **236**, 951-960.

Stadler, H. S., Higgins, K. M. and Capecchi, M. R. (2001). Loss of Eph-receptor expression correlates with loss of cell adhesion and chondrogenic capacity in Hoxa13 mutant limbs. *Development* **128**, 4177-4188.

Svingen, T. and Tonissen, K. F. (2006). Hox transcription factors and their elusive mammalian gene targets. *Heredity* **97**, 88-96.

Sweetman, D. and Munsterberg, A. (2006). The vertebrate spalt genes in development and disease. *Dev. Biol.* **293**, 285-293.

Tabin, C. and Wolpert, L. (2007). Rethinking the proximodistal axis of the vertebrate limb in the molecular era. *Genes Dev.* **21**, 1433-1442.

Tarchini, B., Duboule, D. and Kmita, M. (2006). Regulatory constraints in the evolution of the tetrapod limb anterior-posterior polarity. *Nature* **443**, 985-988.

te Welscher, P., Zuniga, A., Kuijper, S., Drenth, T., Goedemans, H. J., Meijlink, F. and Zeller, R. (2002). Progression of vertebrate limb development through SHH-mediated counteraction of GLI3. *Science* **298**, 827-830.

Toker, A. S., Teng, Y., Ferreira, H. B., Emmons, S. W. and Chalfie, M. (2003). The Caenorhabditis elegans spalt-like gene sem-4 restricts touch cell fate by repressing the selector Hox gene egl-5 and the effector gene mec-3. *Development* **130**, 3831-3840.

Wada, N., Tanaka, H., Ide, H. and Nohno, T. (2003). Ephrin-A2 regulates position-specific cell affinity and is involved in cartilage morphogenesis in the chick limb bud. *Dev. Biol.* **264**, 550-563.

Warren, M., Wang, W., Spiden, S., Chen-Murchie, D., Tannahill, D., Steel, K. P. and Bradley, A. (2007). A Sall4 mutant mouse model useful for studying the role of Sall4 in early embryonic development and organogenesis. *Genesis* **45**, 51-58.

Wilkie, A. O. (2003). Why study human limb malformations? *J. Anat.* **202**, 27-35.

Wilkinson, D. G. (1993). Whole mount in situ hybridization of vertebrate embryos. In *In Situ Hybridization*. Oxford, UK: Oxford University Press.

Williams, M. E., Lehoczky, J. A. and Innis, J. W. (2006). A group 13 homeodomain is neither necessary nor sufficient for posterior prevalence in the mouse limb. *Dev. Biol.* **297**, 493-507.

Yamashita, K., Sato, A., Asashima, M., Wang, P. C. and Nishinakamura, R. (2007). Mouse homolog of SALL1, a causative gene for Townes-Brocks syndrome, binds to A/T-rich sequences in pericentric heterochromatin via its C-terminal zinc finger domains. *Genes Cells* **12**, 171-182.

Yoh, S. M. and Privalsky, M. L. (2001). Transcriptional repression by thyroid hormone receptors. A role for receptor homodimers in the recruitment of SMRT corepressor. *J. Biol. Chem.* **276**, 16857-16867.

Yokouchi, Y., Nakazato, S., Yamamoto, M., Goto, Y., Kameda, T., Iba, H. and Kuroiwa, A. (1995). Misexpression of Hoxa-13 induces cartilage homeotic transformation and changes cell adhesiveness in chick limb buds. *Genes Dev.* **9**, 2509-2522.

Zakany, J. and Duboule, D. (2007). The role of Hox genes during vertebrate limb development. *Curr. Opin. Genet. Dev.* **17**, 359-366.

Zakany, J., Fromental-Ramain, C., Warot, X. and Duboule, D. (1997). Regulation of number and size of digits by posterior Hox genes: a dose-dependent mechanism with potential evolutionary implications. *Proc. Natl. Acad. Sci. USA* **94**, 13695-13700.

Zhu, J., Nakamura, E., Nguyen, M. T., Bao, X., Akiyama, H. and Mackem, S. (2008). Uncoupling Sonic hedgehog control of pattern and expansion of the developing limb bud. *Dev. Cell* **14**, 624-632.

Zuniga, A., Haramis, A. P., McMahon, A. P. and Zeller, R. (1999). Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* **401**, 598-602.
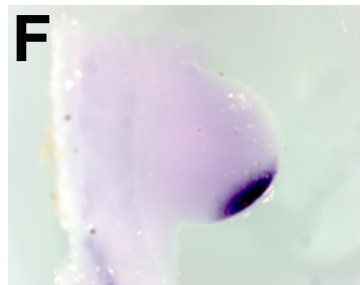
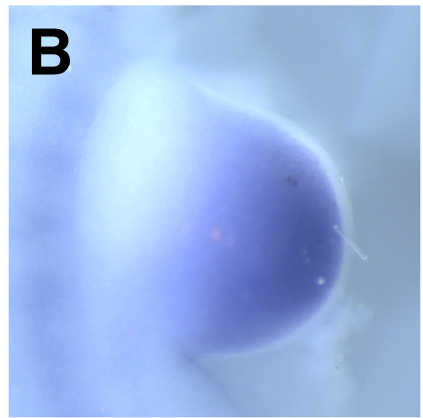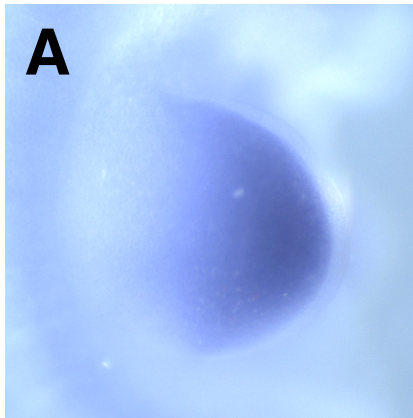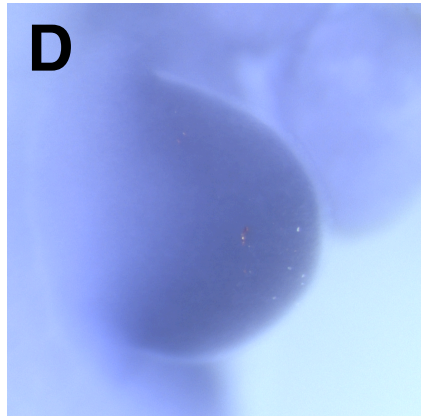WT   *Sall1^{-/-}; Sall3^{-/-}*

Fgf8
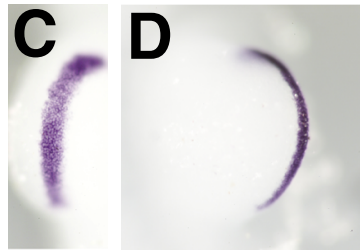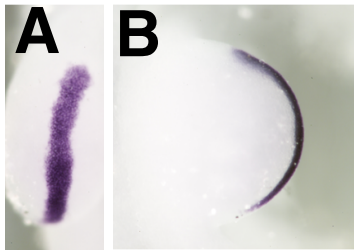
Shh

	Control	$Sall1^{-/-}; Sall3^{-/-}$
Sall4	A	B
Tbx5	C	D
Tbx4	E	F
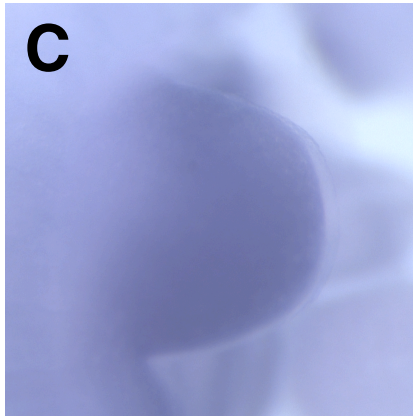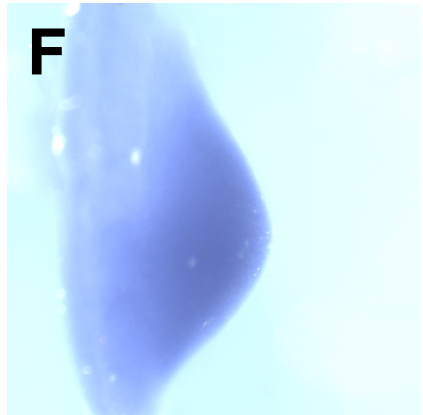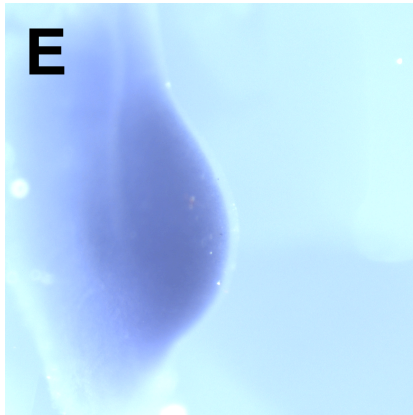
Tbx5^+/+ ... _Tbx5_^+/-
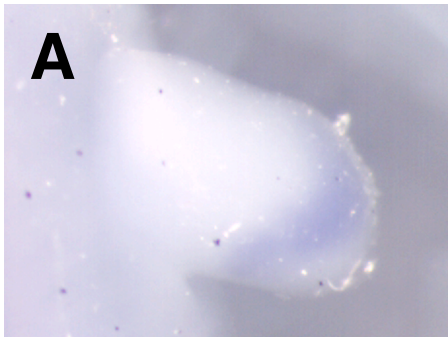
	Tbx5+/+	Tbx5+/-
Sall1	A	B
Sall3	C	D
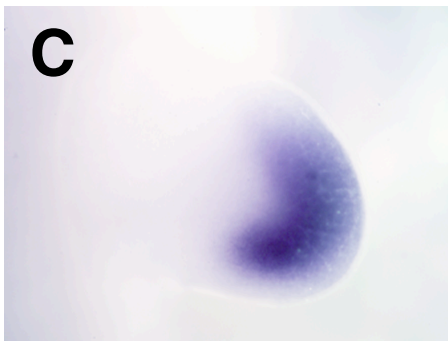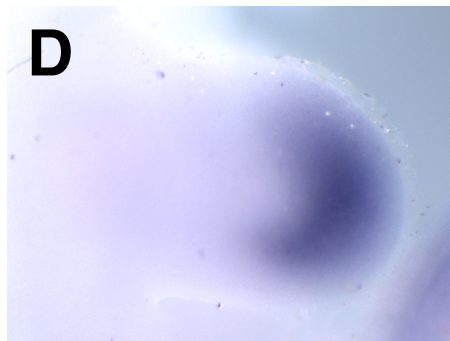
WT   *Sall1^{-/-}; Sall3^{-/-}*

Hoxa11
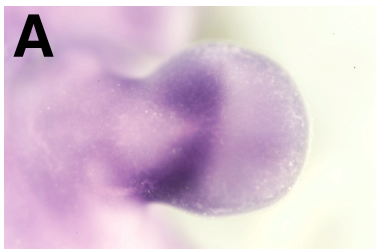
A   B
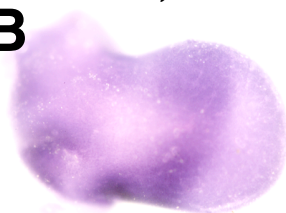
Hoxa13
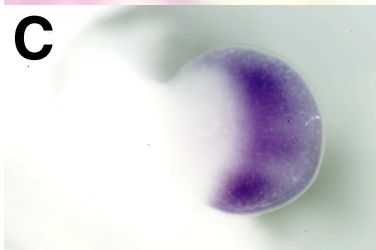
C   D
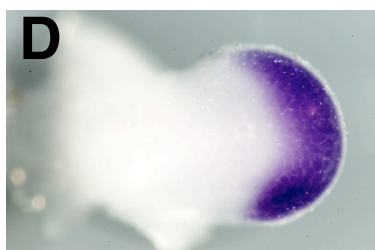
Hoxd11

E   F

Hoxd12

G   H

Hoxd13

I   J